Total Effective Xenoestrogen Burden in Serum Samples and Risk for Breast Cancer in a Population-Based Multicase-Control Study in Spain

Roberto Pastor-Barriuso, Mariana F. Fernández,
Gemma Castaño-Vinyals, Denis Whelan, Beatriz Perez-Gomez,
Javier Llorca, Cristina M. Villanueva, Marcela Guevara,
Jose-Manuel Molina Molina, Francisco Artacho-Cordon,
Laura Barriuso-Lapresa, Ignasi Tusquets,
Trinidad Dierssen-Sotos, Nuria Aragonés, Nicolás Olea,
Manolis Kogevinas, and Marina Pollan

Note to readers with disabilities: *EHP* will provide a 508-conformant version of this article upon final publication. If you require a 508-conformant version before then, please contact ehp508@niehs.nih.gov. Our staff will work with you to assess and meet your accessibility needs within 3 working days.

NIH National Institute of Environmental Health Sciences

Total Effective Xenoestrogen Burden in Serum Samples and Risk for Breast Cancer in a Population-Based Multicase-Control Study in Spain

Roberto Pastor-Barriuso,[1,2]* Mariana F. Fernández,[2,3]* Gemma Castaño-Vinyals,[2,4,5,6] Denis Whelan,[1,7,8] Beatriz Perez-Gomez,[1,2] Javier Llorca,[2,9] Cristina M. Villanueva,[2,4,5,6] Marcela Guevara,[2,10] Jose-Manuel Molina Molina,[3] Francisco Artacho-Cordon,[3] Laura Barriuso-Lapresa,[2,10] Ignasi Tusquets,[5,11,12] Trinidad Dierssen-Sotos,[2,9] Nuria Aragonés,[1,2] Nicolás Olea,[2,3] Manolis Kogevinas,[2,4,5,6] and Marina Pollan[1,2]

[1]National Center for Epidemiology, Carlos III Institute of Health, Madrid, Spain; [2]Consortium for Biomedical Research in Epidemiology and Public Health (CIBERESP), Madrid, Spain; [3]Biosanitary Institute of Granada (ibs.GRANADA), University of Granada, Granada, Spain; [4]Center for Research in Environmental Epidemiology (CREAL), Barcelona, Spain; [5]Hospital del Mar Medical Research Institute (IMIM), Barcelona, Spain; [6]Pompeu Fabra University (UPF), Barcelona, Spain; [7]USA-Spain Fulbright Commission for Cultural, Educational, and Scientific Exchange, Madrid, Spain; [8]Department of Biostatistics and Bioinformatics, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA; [9]Division of Epidemiology and Computational Biology, School of Medicine, University of Cantabria, Santander, Spain; [10]Public Health Institute of Navarra, Pamplona, Spain; [11]Medicine Department, Autonomous University of Barcelona, Barcelona, Spain; [12]Medical Oncology Department, Hospital del Mar, Barcelona, Spain. *These authors contributed equally to this work.

1

Address correspondence to M. Pollán, National Center for Epidemiology, Carlos III Institute of

Health, Monforte de Lemos 5, 28029 Madrid, Spain. Telephone: +34 91 822 26 35. E-mail:

mpollan@isciii.es

Running title: Serum total xenoestrogen burden and breast cancer

Competing financial interests: The authors declare they have no actual or potential competing

financial interests.

Abstract

Background: Most studies on endocrine disrupting chemicals and breast cancer have focused on single compounds with inconclusive findings.

Objectives: We assessed the combined estrogenic effect of mixtures of xenoestrogens in serum and its relation to breast cancer risk.

Methods: A total of 186 incident pretreatment breast cancer cases and 196 frequency-matched controls were randomly sampled from a large population-based multicase-control study in Spain. The total effective xenoestrogen burden attributable to organohalogenated xenoestrogens (TEXB-α) and endogenous hormones and more polar xenoestrogens (TEXB-β) were determined in serum samples by using high-performance liquid chromatography separation and E-Screen bioassay. Odds ratios for breast cancer comparing tertiles of serum TEXB-α and TEXB-β were estimated using logistic models, and smooth risk trends using spline models.

Results: Cases had higher geometric mean TEXB-α and TEXB-β levels (8.32 and 9.94 Eeq pM/mL) than controls (2.99 and 5.96 Eeq pM/mL, respectively). The fully-adjusted odds ratios for breast cancer (95% confidence intervals) comparing the second and third tertiles of TEXB-α with the first tertile were 1.77 (0.76, 4.10) and 3.45 (1.50, 7.97), and those for TEXB-β were 2.35 (1.10, 5.03) and 4.01 (1.88, 8.56). A steady increase in risk was evident across all detected TEXB-α levels and a sigmoidal trend for TEXB-β. Individual xenoestrogens showed weak and opposed associations with breast cancer risk.

Conclusions: This is the first study to show a strong positive association between serum total xenoestrogen burden and breast cancer risk, thus highlighting the importance of evaluating xenoestrogen mixtures, rather than single compounds, when studying hormone-related cancers.

Introduction

Malignant breast tumors are the leading cause of cancer in women worldwide in terms of

incidence and mortality (Ferlay et al. 2013). In spite of efforts to elucidate breast cancer etiology,

genetic determinants and well-established risk factors explain a limited amount of the global

burden of this disease (Barnes et al. 2011; Howell et al. 2014; Sprague et al. 2008). It is

noteworthy that most recognized determinants of breast cancer, such as reproductive history,

alcohol intake, obesity, and use of hormone therapy, exert their effect, at least in part, by

modifying the time and intensity of the exposure of the mammary gland to steroidal hormones

(Brown and Hankinson 2015; Hilakivi-Clarke et al. 2013; MacMahon 2006; Renehan et al. 2015;

Seitz et al. 2012).

Laboratory studies, specifically rodent models, support the implication of environmental

pollutants in breast cancer development (Dhimolea et al. 2014; Rudel et al. 2007). Endocrine

disrupting chemicals (EDCs) are among the 17 chemical groups prioritized for evaluation in

epidemiological studies on breast cancer (Rudel et al. 2014) due to their potential to act as

xenoestrogens or modulate the estrogenic activity via different pathways (Gibson and Saunders

2014; WHO/UNEP 2013). Hundreds of EDCs are present in human breast tissue, but

epidemiological evidence linking these substances with breast cancer is inconclusive

(WHO/UNEP 2013). Most previous studies have focused on individual EDCs with weak

estrogenic effects, thus failing to consider multiple exposures and interactions involving different

EDCs and physiological hormones (Fernandez et al. 2014). Functional tests measuring the

combined estrogenic activity of mixtures of EDCs offer a promising approach for an aggregated

exposure assessment. A case-control study reported a positive association between the combined

effect of environmental estrogens in human adipose tissue and breast cancer risk (Ibarluzea et al.

2004). Adipose tissue, however, is difficult to obtain in population-based studies, and it would be

of great practical value to assess the estrogenic potential of EDC mixtures present in blood

samples (Rudel et al. 2014).

In the present study, we measured the combined estrogenic activity of mixtures of

xenoestrogens in serum samples and evaluated its relation to breast cancer risk in a subsample of

cases and controls from a large population-based multicase-control study in Spain (MCC-Spain).

Methods

Study population. MCC-Spain (http://www.mccspain.org) is a population-based multicase-

control study conducted between 2008–2013 in 12 Spanish provinces to identify environmental,

personal, and genetic factors related to five common cancers, including breast, prostate,

colorectal, stomach, and chronic lymphocytic leukemia. The study design has been previously

reported (Castano-Vinyals et al. 2015). Briefly, the study recruited 6,082 histologically

confirmed incident cancer cases aged 20–85 years, including 1,750 breast cancers, 1,115 prostate

cancers, 2,171 colorectal cancers, 492 gastro-oesophageal cancers, and 554 cases of leukemia, as

well as a single set of 4,101 population controls. The response rates were 69% among breast

cancer cases and 54% among female controls. All participants completed computer-assisted

personal interviews on sociodemographic factors, self-reported anthropometric data, lifestyle,

reproductive history, hormonal factors, medications, and personal and family medical history.

Blood samples were collected from 76% of participants. The study was approved by the ethics

committees of the participating institutions. Written informed consent was obtained from each

participant.

For this analysis, we randomly selected 204 breast cancer cases among those who agreed to

donate blood samples in the provinces of Madrid, Barcelona, Navarra, and Cantabria and 204

female controls frequency-matched to cases by province, 5-year age interval, and 2-unit category
of body mass index.

Biochemical analyses. We measured the total effective xenoestrogen burden (TEXB) in serum

samples by using a standardized bioassay for the combined estrogenic effect of mixtures of

xenoestrogens (Fernandez et al. 2004), which has been applied to extracts of adipose tissue,

blood, and placenta (Fernandez et al. 2007; Ibarluzea et al. 2004; Lopez-Espinosa et al. 2009;

Sonnenschein et al. 1995). Three mL of serum were added to the same volume of methanol and

the solution was extracted with 5 mL of hexane/ethyl ether (1:1 v/v). The organic phase was then

passed through a Bond Elut PCB cartridge (Varian), previously conditioned with 1.5 mL hexane.

The eluate obtained was dried at reduced pressure under a stream of nitrogen. Serum dried

extracts were reconstituted in 200 µL hexane, halved, and eluted in duplicate by high-

performance liquid chromatography (HPLC). This semipreparative HPLC method was

developed to efficiently separate organohalogenated lipophilic xenoestrogens [organochlorine

pesticides and metabolites, polychlorinated biphenyls (PCBs), and halogenated bisphenols,

among others] eluting in the alpha fraction from endogenous hormones and more polar

xenoestrogens (non-halogenated bisphenols, polyphenols, phytoestrogens, and mycoestrogens)

eluting in the beta fraction, using a normal-phase column and a gradient with two mobile phases

[n-hexane (phase A) and n-hexane:methanol:2-isopropanol (40:45:15 v/v) (phase B)], with the

most lipophilic compounds eluting in the shortest time. After HPLC fractionation, duplicated dry

extracts of each fraction were joined, resuspended in experimental steroid-free medium (phenol

red-free medium, supplemented with 2.5 mL of charcoal-dextran fetal bovine serum), and tested

for estrogenic activity in the E-Screen bioassay (Soto et al. 1995). The combined estrogenic

activity of all compounds included in each fraction was analyzed from its proliferative effect on

MCF-7 human breast cancer cells. Each fraction extract was assayed at three different dilutions (1:1, 1:5, and 1:10), together with a negative control (experimental steroid-free medium) and a positive control (treated with 100 pM of estradiol) in each culture plate. The proliferative effects of alpha and beta fractions were calculated as the difference in MCF-7 cell proliferation between the fraction extract and the steroid-free control, divided by the highest difference in proliferation between the estradiol-treated and steroid-free control cells. These relative proliferative effects were transformed into estradiol equivalent units by reading from a sigmoidal dose-response curve prepared with estradiol at concentrations of 0.1–1000 pM, and they were expressed as the estradiol equivalent concentration in picomolar per milliliter of serum (Eeq pM/mL) that would produce the same cell proliferation in the bioassay (Fernandez et al. 2007). Thus, TEXB of the alpha fraction (TEXB-α) can be regarded as a biomarker of the combined estrogenic effect of mixtures of organohalogenated lipophilic xenoestrogens, whereas TEXB of the beta fraction (TEXB-β) represents the combined estrogenic activity of endogenous hormones and more polar xenoestrogens.

The limit of detection for TEXB-α and TEXB-β was 0.1 Eeq pM/mL, which corresponded to the minimum concentration needed to produce a significantly different proliferative effect from that observed in steroid-free control cells. For 6.3% and 3.7% of participants with TEXB-α and TEXB-β determinations below the limit of detection, a level equal to the limit of detection divided by the square root of 2 was imputed. TEXB-α and TEXB-β levels could not be determined in 44.2% and 35.1% of serum samples, respectively, because MCF-7 cells treated with their extracts grew less than steroid-free control cells, which hampered reading the proliferative effect in the estradiol dose-response curve. For quality control, 10 serum samples were analyzed in triplicate through independent extraction, HPLC fractionation, and E-Screen

bioassay. The interassay coefficients of variation for TEXB-α and TEXB-β were 18.5% and

11.1%, respectively.

Specific organohalogenated compounds present in the alpha HPLC fraction, such as PCB-

138, PCB-153, PCB-180, hexachlorobenzene (HCB), and *p,p*'-dichlorodiphenyldichloroethylene

(*p,p*'-DDE), were quantified by high-resolution gas chromatography with micro-electron capture

detection, using *p*-chlorodibenzophenone as internal standard. The limit of detection for all these

chemicals was set at 0.05 ng/mL, representing the smallest analyte amount that gave a signal-to-

noise ratio greater than 3. For 5.2%, 1.8%, 3.4%, 8.4%, and 2.1% of participants with serum

concentrations of PCB-138, PCB-153, PCB-180, HCB, and *p,p*'-DDE below the limit of

detection, respectively, a level equal to the limit of detection divided by the square root of 2 was

imputed. Total cholesterol and triglycerides were enzymatically quantified in 10 μL of serum

using a Cobas 400 analyzer (Roche), and total lipids were derived from the short formula based

on these measured lipid species (Phillips et al. 1989).

Statistical analysis. Participants were grouped into tertiles of serum TEXB-α and TEXB-β levels

based on their distributions among controls. Odds ratios for breast cancer and 95% confidence

intervals (CIs) comparing the second and third tertiles with the first tertile of serum TEXB-α and

TEXB-β were estimated using logistic regression models. We also estimated the odds ratio for

women with undetermined estrogenicity in the bioassay compared with all other women with

determined estrogenicity. Tests for linear risk trend across serum TEXB-α and TEXB-β tertiles

were performed by including an ordinal variable with the median level of each tertile among

controls in logistic regression models. To further explore the shape of the dose-response relations

of serum TEXB-α and TEXB-β levels with breast cancer risk, we used restricted quadratic

splines for log-transformed TEXB-α and TEXB-β levels with knots at the 10th, 50th, and 95th

percentiles of their control distributions (the first knot was set at the 10th percentile to exceed

levels below the limit of detection) (Greenland 1995). We also estimated odds ratios for breast

cancer comparing tertiles of specific organohalogenated compounds (PCB-138, PCB-153, PCB-

180, HCB, and *p,p*'-DDE) based on their control distributions.

Logistic regression models were fitted with increasing degrees of adjustment. The first model

adjusted for province (Madrid, Barcelona, Navarra, or Cantabria), age (continuous), body mass

index (continuous), education level (primary or less, high school, or college), and serum total

lipid levels (continuous). The second model further adjusted for breast cancer risk factors,

including smoking status (never, former, or current), number of births (nulliparous, 1–2, or ≥ 3),

age at first birth (continuous), menopausal status (premenopausal or postmenopausal), use of

hormone replacement therapy (never or ever), previous breast biopsy (no or yes), and family

history of breast cancer (no, second-degree relative, or first-degree relative). Finally, the third

model mutually adjusted serum TEXB-α and TEXB-β levels for each other. Effect modifications

were contrasted by including interaction terms of serum TEXB-α and TEXB-β tertiles with each

of the above covariates in logistic regression models. Analyses were performed using Stata,

version 13.1 (StataCorp) and R, version 2.15 (R Foundation for Statistical Computing).

Results

From the 408 randomly selected women (204 breast cancer cases and 204 controls), we excluded

5 prevalent or recurrent cases of breast cancer at baseline interview, 5 cases who initiated chemo

or hormone therapy before blood extraction, one case who withdrew initial consent, and 15

additional women (7 cases and 8 controls) with insufficient serum samples. Thus, the final

sample included 186 incident pretreatment cases of breast cancer (166 invasive and 20 ductal

carcinoma in situ) and 196 population-based controls with available serum samples for

estrogenicity analyses. The mean age and body mass index among cases and frequency-matched

controls were 59.8 years and 26.3 kg/m^2, respectively. Compared with controls, cases were more

likely to be nulliparous, ever smokers, ever users of hormone therapy, have lower education

level, and have higher prevalences of breast biopsies and affected first-degree relatives, though

only the difference in the prevalence of breast biopsies was statistically significant (Table 1). The

geometric mean serum levels of TEXB-α and TEXB-β were significantly higher in breast cancer

cases (8.32 and 9.94 Eeq pM/mL) than in controls (2.99 and 5.96 Eeq pM/mL, respectively).

Samples with undetermined estrogenicity in the bioassay were equally distributed among cases

and controls. Regarding specific organohalogenated compounds, cases had marginally lower

HCB concentrations and similar levels of PCB-138, PCB-153, PCB-180, and *p,p'*-DDE than

controls (Table 1).

Serum levels of TEXB-α and TEXB-β were moderately correlated among controls (Pearson

correlation coefficient for log-transformed variables of 0.34; 95% CI: 0.14, 0.51). Serum

concentrations of PCB-138, PCB-153, PCB-180, HCB, and *p,p'*-DDE were weakly correlated

with TEXB-α levels among controls (Pearson correlations for log-transformed variables of -0.21,

-0.01, -0.18, -0.18, and -0.17, respectively) and virtually uncorrelated with TEXB-β levels (-0.05,

-0.03, -0.07, -0.02, and 0.03). Apart from differences by geographic region, no other significant

trend in breast cancer risk factors or serum chemical concentrations was observed across tertiles

of serum TEXB-α and TEXB-β levels among controls, partially due to the limited number of

control women within each tertile (Table 2). Compared with controls with determined

estrogenicity in serum samples, controls with undetermined TEXB-α and TEXB-β had similar

risk factor distributions, but significantly higher serum concentrations of PCB-138, PCB-180,

HCB, and *p,p'*-DDE (Table 2).

In models adjusted for sociodemographic and traditional breast cancer risk factors (Table 3), the risk for breast cancer increased with increasing serum levels of both TEXB-α and TEXB-β (p for linear trend = 0.003 and 0.001, respectively). Compared with the first tertile, the odds ratios for the second and third tertiles of serum TEXB-α were 1.77 (95% CI: 0.76, 4.10) and 3.45 (95% CI: 1.50, 7.97), and those for the second and third tertiles of serum TEXB-β were 2.35 (95% CI: 1.10, 5.03) and 4.01 (95% CI: 1.88, 8.56). The increase in breast cancer risk was marked and sustained over all serum TEXB-α levels above 0.5 Eeq pM/mL (Figure 1A). However, a sigmoidal risk trend was observed across serum TEXB-β levels, with a sharp increase in risk between 2–40 Eeq pM/mL and a downturn at higher levels (Figure 1B). When serum TEXB-α and TEXB-β levels were mutually adjusted for each other, the association of TEXB-α with breast cancer risk was substantially attenuated, while that for TEXB-β remained virtually unchanged (Table 3 and Figure 1). The risk for breast cancer did not differ among women with undetermined TEXB-α and TEXB-β compared with women with determined estrogenicity (fully-adjusted odds ratios of 0.73 and 0.97, respectively) (Table 3).

Individual organohalogenated xenoestrogens contained in the alpha fraction showed weak and opposed associations with breast cancer risk (Table 4). In models adjusted for sociodemographic and traditional risk factors, the odds ratios for breast cancer comparing the third with the first tertile were 1.73 (95% CI: 0.96, 3.14) for PCB-138, 1.36 (95% CI: 0.75, 2.45) for PCB-153, 1.01 (95% CI: 0.55, 1.87) for PCB-180, 0.84 (95% CI: 0.45, 1.58) for p,p'-DDE, and 0.60 (95% CI: 0.32, 1.15) for HCB.

In subgroup analyses, the increased risk for breast cancer in the third versus the first tertile of serum TEXB-α tended to be higher in women with normal weight and those with family history of breast cancer (subgroup-specific odds ratios of 6.37 and 5.78, respectively), although none of

these effect modifications was statistically significant (Figure 2). The positive association of

serum TEXB-β with breast cancer risk was quite homogeneous across all subgroups.

Discussion

This is the first study showing a graded positive association between serum total xenoestrogen

burden, as determined by the alpha fraction of the TEXB bioassay, and breast cancer risk.

Women in the third tertile of serum TEXB-α had a 3.45-fold increase in breast cancer risk

compared with those in the first tertile. The beta fraction of the TEXB bioassay was also

positively associated with the risk for breast cancer, a result somewhat expected taking into

account that circulating endogenous estrogens are included in this fraction. Finally, none of the

individual organohalogenated xenoestrogens analyzed in this study was significantly associated

with breast cancer risk.

Most studies in this field have focused on serum or adipose concentrations of a single

chemical or a reduced number of chemicals, ignoring the cumulative effects of mixtures.

Recognizing this limitation, the World Health Organization Report has concluded that it is

critical to move beyond the analysis of one chemical at a time to explore the effects of EDC

mixtures (WHO/UNEP 2013). The TEXB bioassay is an alternative technique that directly

measures the combined estrogenic effect of all compounds included in either alpha or beta HPLC

fractions. Since additive, synergistic, or antagonistic mechanisms may be present in these

complex mixtures (Evans et al. 2012; Scholze et al. 2014), this approach constitutes a more

efficient way to explore the cumulative impact of these compounds. In fact, cell culture studies

have shown that EDC mixtures can produce a significant proliferative effect even at

concentrations of individual chemicals that alone do not produce detectable effects (Rajapakse et

al. 2002). Thus, the estrogenic potential of EDCs, when tested individually, is likely to be

underestimated (Kortenkamp 2007).

Most women in this study (83.5%) had detectable serum concentrations of all the measured

organochlorine chemicals, reflecting the ubiquity of their exposure in our population. We found

no differences in PCB, HCB, or *p,p*'-DDE concentrations between cases and controls, and none

of these single compounds was positively correlated with serum TEXB-α levels among controls,

reflecting their modest contribution to the total xenoestrogen burden. Similarly, a previous study

has reported no correlation between individual organohalogenated xenoestrogens and their

combined estrogenic activity in adipose tissue (Ibarluzea et al. 2004).

The extensive HPLC fractionation previous to the TEXB bioassay was designed to separate

organohalogenated lipophilic xenoestrogens in the alpha fraction from endogenous hormones

and more polar xenoestrogens in the beta fraction (Fernandez et al. 2004). In our study, serum

TEXB-α and TEXB-β levels were positively correlated among controls, which resulted in an

attenuation of the association between TEXB-α and breast cancer risk after adjusting for TEXB-

β. The causal diagram of Figure 3 displays the assumed causal relations among TEXB-α, TEXB-

β, breast cancer, and other relevant exposures, which provides a valuable tool for identifying

potential sources of bias and their control. Serum TEXB-α and TEXB-β levels are assumed to be

affected by an unspecified common exposure to both lipophilic and polar xenoestrogens

(Fernandez et al. 2004), as suggested by the observed association between TEXB-α and TEXB-

β. Serum TEXB-β levels are also affected by unmeasured endogenous hormones, which are

independent of xenoestrogen exposure and directly influence breast cancer risk. According to

this diagram, the causal effect of TEXB-α on breast cancer is confounded by correlated

xenoestrogens present in the beta fraction, whose upward bias can be controlled by adjusting for

TEXB-β. However, this adjustment induces a negative conditional association between xenoestrogens and endogenous hormones, which results in a downward selection bias that can be as severe as the controlled confounding if endogenous hormone effects are strong (Greenland 2003). Thus, without further information on xenoestrogen exposure or endogenous hormones, we can just conclude that the underlying effect of TEXB-α on breast cancer lies between the estimated associations with and without adjustment for TEXB-β.

While the risk for breast cancer increased progressively across all detected TEXB-α levels above 0.5 Eeq pM/mL, the association for TEXB-β followed a sigmoidal trend, with a sharp increase in risk between 2–40 Eeq pM/mL and a downturn at higher levels. Nonmonotonic responses are remarkably common in studies of natural hormones and EDCs (Engstrom et al. 2015; Vandenberg et al. 2012). The upward-then-downward risk trend for TEXB-β could be explained by receptor competition between endogenous hormones and polar xenoestrogens included in the beta fraction (Vandenberg et al. 2012). At low-to-intermediate TEXB-β levels, natural hormone concentrations do not saturate receptors and xenoestrogens bind to unoccupied receptors to increase the overall cellular response; but at high TEXB-β levels, xenoestrogens can outcompete natural ligands and, due to their weaker estrogenic activity, result in an attenuation of the overall biological response.

Though the study had limited power to detect effect modifications, we observed somewhat higher effects of TEXB-α on breast cancer risk in women with normal weight and those with family history of breast cancer. A stronger effect in leaner women was also evident in a previous case-control study using the TEXB bioassay in adipose tissue (Ibarluzea et al. 2004), which was attributed to a greater relative impact of EDCs in women with lower levels of endogenous hormones accumulated in their fat. Regarding the stronger association between TEXB-α and

breast cancer risk in women with family history of breast cancer, this group was too small to

draw further conclusions and larger studies are needed to confirm this potential effect-measure

modification.

Contrary to previous findings in adipose tissue extracts (Fernandez et al. 2004; Ibarluzea et al.

2004), the combined estrogenic activity in serum samples was not associated with age, body

mass index, or any other women characteristic in our study, with the exception of the differences

observed by geographical region. Control women from the province of Navarra had higher

TEXB-α and TEXB-β levels, while those from Cantabria presented lower estrogenic activity in

both fractions. We have no clear explanation for these geographical differences. Previous studies

have reported higher serum concentrations of PCBs in healthy adults from the northern Spanish

regions (Agudo et al. 2009; Huetos et al. 2014) and elevated HCB levels in the province of

Navarra (Jakszyn et al. 2009). However, these single chemicals contributed little to the total

xenoestrogen burden in women of this study and can hardly explain the observed regional

variations. Navarra is also one of the Spanish regions with higher prevalence of postmenopausal

hormone therapy use (Isidoro et al. 2015), but our study found no differences in TEXB-α or

TEXB-β levels between never and ever users of hormone therapy. Thus, larger population-based

studies are required to identify determinants of serum TEXB levels that contribute to explain

their geographical distribution.

The strengths of this study include the population-based case-control design and the use of a

reliable biomarker for the combined estrogenic effect of EDC mixtures. However, several

limitations must be mentioned. First, the response rate among population controls was moderate,

with higher participation rates among women with higher education level. To control for this

potential selection bias, all analyses were adjusted for education level. Second, owing to the

case-control design, serum samples were collected after diagnosis in breast cancer cases, which

might have led to a reverse causation bias if serum concentrations of hormones or xenoestrogens

had changed after disease onset. To minimize the potential for reverse causation, we restricted

the analysis to incident cases of breast cancer who did not receive neoadjuvant chemo or

hormone therapy before blood extraction. However, as most growing breast tumors are estrogen-

demanding, serum estrogen levels might have decreased after disease onset, leading to a

potential dilution in the associations, particularly for TEXB-β since endogenous hormones have

higher binding affinity to estrogen receptors and shorter biological half-lives than xenoestrogens.

Third, adipose tissue extracts were not collected in our study and TEXB in serum samples was

taken as a surrogate of the overall estrogenic activity at the mammary gland. While many EDCs

are lipophilic and accumulate in the breast fatty tissue, their concentrations in serum are

relatively low and depend on serum lipid content. For this reason, all analyses relating serum

TEXB levels with breast cancer risk were adjusted for serum total lipids. Fourth, estrogenicity

could not be determined in over one third of serum samples because breast cancer cells treated

with their extracts grew less than steroid-free control cells in the TEXB bioassay. Although there

is no clear explanation for this lack of growth, samples with undetermined estrogenicity had

significantly higher levels of all measured organohalogenated xenoestrogens, so that they might

also have elevated concentrations of other unmeasured common-source xenobiotics that

prevented or hampered cellular growth. Nevertheless, since undetermined estrogenicity was

unrelated to case-control status, our analyses based on determined samples will provide an

unbiased estimate of the association between TEXB and breast cancer risk. Finally, the inherent

time-consuming and serum-demanding characteristics of the TEXB bioassay, together with the

substantial proportion of undetermined samples, heavily limited the effective sample size and

power of the present study, which precluded more extensive analyses according to tumor subtypes.

Conclusions

The combined estrogenic activity of mixtures of organohalogenated xenoestrogens in serum samples was positively associated with breast cancer risk, even though no single compound showed a significant effect when analyzed separately. The increase in risk was strong and progressive across all detected estrogenic levels. Our findings show the importance of evaluating mixtures of EDCs, rather than single chemicals, in epidemiological studies on hormone-related cancers. This study provides new evidence linking breast cancer with combined exposures to EDCs to be considered by policy agencies in charge of controlling their production and distribution.

References

Agudo A, Goni F, Etxeandia A, Vives A, Millan E, Lopez R, et al. 2009. Polychlorinated

biphenyls in Spanish adults: determinants of serum concentrations. Environ Res 109:620-628.

Barnes BB, Steindorf K, Hein R, Flesch-Janys D, Chang-Claude J. 2011. Population attributable

risk of invasive postmenopausal breast cancer and breast cancer subtypes for modifiable and

non-modifiable risk factors. Cancer Epidemiol 35:345-352.

Brown SB, Hankinson SE. 2015. Endogenous estrogens and the risk of breast, endometrial, and

ovarian cancers. Steroids 99:8-10.

Castano-Vinyals G, Aragones N, Perez-Gomez B, Martin V, Llorca J, Moreno V, et al. 2015.

Population-based multicase-control study in common tumors in Spain (MCC-Spain): rationale

and study design. Gac Sanit 29:308-315.

Dhimolea E, Wadia PR, Murray TJ, Settles ML, Treitman JD, Sonnenschein C, et al. 2014.

Prenatal exposure to BPA alters the epigenome of the rat mammary gland and increases the

propensity to neoplastic development. PLoS One 9:e99800;

doi:10.1371/journal.pone.0099800.

Engstrom W, Darbre P, Eriksson S, Gulliver L, Hultman T, Karamouzis MV, et al. 2015. The

potential for chemical mixtures from the environment to enable the cancer hallmark of

sustained proliferative signalling. Carcinogenesis 36(suppl 1):S38-S60.

Evans RM, Scholze M, Kortenkamp A. 2012. Additive mixture effects of estrogenic chemicals

in human cell-based assays can be influenced by inclusion of chemicals with differing effect

profiles. PLoS One 7:e43606; doi:10.1371/journal.pone.0043606.

Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, et al. 2013. GLOBOCAN

2012, Cancer Incidence and Mortality Worldwide. IARC CancerBase No. 11, version 1.0.

Available: http://globocan.iarc.fr [accessed 7 January 2016].

Fernandez MF, Rivas A, Olea-Serrano F, Cerrillo I, Molina-Molina JM, Araque P, et al. 2004.

Assessment of total effective xenoestrogen burden in adipose tissue and identification of

chemicals responsible for the combined estrogenic effect. Anal Bioanal Chem 379:163-170.

Fernandez MF, Roman M, Arrebola JP, Olea N. 2014. Endocrine disruptors: time to act. Curr

Environ Health Rep 1:325-332.

Fernandez MF, Santa-Marina L, Ibarluzea JM, Exposito J, Aurrekoetxea JJ, Torne P, et al. 2007.

Analysis of population characteristics related to the total effective xenoestrogen burden: a

biomarker of xenoestrogen exposure in breast cancer. Eur J Cancer 43:1290-1299.

Gibson DA, Saunders PT. 2014. Endocrine disruption of oestrogen action and female

reproductive tract cancers. Endocr Relat Cancer 21:T13-T31.

Greenland S. 1995. Dose-response and trend analysis in epidemiology: alternatives to categorical

analysis. Epidemiology 6:356-365.

Greenland S. 2003. Quantifying biases in causal models: classical confounding vs collider-

stratification bias. Epidemiology 14:300-306.

Hilakivi-Clarke L, de Assis S, Warri A. 2013. Exposures to synthetic estrogens at different times

during the life, and their effect on breast cancer risk. J Mammary Gland Biol Neoplasia 18:25-

42.

Howell A, Anderson AS, Clarke RB, Duffy SW, Evans DG, Garcia-Closas M, et al. 2014. Risk

determination and prevention of breast cancer. Breast Cancer Res 16:446.

Huetos O, Bartolome M, Aragones N, Cervantes-Amat M, Esteban M, Ruiz-Moraga M, et al. 2014. Serum PCB levels in a representative sample of the Spanish adult population: the BIOAMBIENT.ES project. Sci Total Environ 493:834-844.

Ibarluzea JJ, Fernandez MF, Santa-Marina L, Olea-Serrano MF, Rivas AM, Aurrekoetxea JJ, et al. 2004. Breast cancer risk and the combined effect of environmental estrogens. Cancer Causes Control 15:591-600.

Isidoro B, Lope V, Whelan D, Pedraz C, Sanchez-Contador C, Santamarina C, et al. 2015. Use of hormone therapy and isoflavones and mammographic density in Spain. Menopause; doi:10.1097/gme.0000000000000569 [Online 28 December 2015].

Jakszyn P, Goni F, Etxeandia A, Vives A, Millan E, Lopez R, et al. 2009. Serum levels of organochlorine pesticides in healthy adults from five regions of Spain. Chemosphere 76:1518-1524.

Kortenkamp A. 2007. Ten years of mixing cocktails: a review of combination effects of endocrine-disrupting chemicals. Environ Health Perspect 115(suppl 1):98-105.

Lopez-Espinosa MJ, Silva E, Granada A, Molina-Molina JM, Fernandez MF, Aguilar-Garduno C, et al. 2009. Assessment of the total effective xenoestrogen burden in extracts of human placentas. Biomarkers 14:271-277.

MacMahon B. 2006. Epidemiology and the causes of breast cancer. Int J Cancer 118:2373-2378.

Phillips DL, Pirkle JL, Burse VW, Bernert JT, Henderson LO, Needham LL. 1989. Chlorinated hydrocarbon levels in human serum: effects of fasting and feeding. Arch Environ Contam Toxicol 18:495-500.

Rajapakse N, Silva E, Kortenkamp A. 2002. Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action. Environ Health Perspect 110:917-921.

Renehan AG, Zwahlen M, Egger M. 2015. Adiposity and cancer risk: new mechanistic insights from epidemiology. Nat Rev Cancer 15:484-498.

Rudel RA, Ackerman JM, Attfield KR, Brody JG. 2014. New exposure biomarkers as tools for breast cancer epidemiology, biomonitoring, and prevention: a systematic approach based on animal evidence. Environ Health Perspect 122:881-895.

Rudel RA, Attfield KR, Schifano JN, Brody JG. 2007. Chemicals causing mammary gland tumors in animals signal new directions for epidemiology, chemicals testing, and risk assessment for breast cancer prevention. Cancer 109:2635-2666.

Scholze M, Silva E, Kortenkamp A. 2014. Extending the applicability of the dose addition model to the assessment of chemical mixtures of partial agonists by using a novel toxic unit extrapolation method. PLoS One 9:e88808; doi:10.1371/journal.pone.0088808.

Seitz HK, Pelucchi C, Bagnardi V, La Vecchia C. 2012. Epidemiology and pathophysiology of alcohol and breast cancer: update 2012. Alcohol Alcohol 47:204-212.

Sonnenschein C, Soto AM, Fernandez MF, Olea N, Olea-Serrano MF, Ruiz-Lopez MD. 1995. Development of a marker of estrogenic exposure in human serum. Clin Chem 41:1888-1895.

Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. 1995. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect 103(suppl 7):113-122.

Sprague BL, Trentham-Dietz A, Egan KM, Titus-Ernstoff L, Hampton JM, Newcomb PA. 2008.

Proportion of invasive breast cancer attributable to risk factors modifiable after menopause.

Am J Epidemiol 168:404-411.

Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Lee DH, et al. 2012. Hormones

and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses.

Endocr Rev 33:378-455.

WHO/UNEP (World Health Organization/United Nations Environment Programme). 2013. State

of the Science of Endocrine Disrupting Chemicals - 2012. Available:

http://www.who.int/ceh/publications/endocrine/en/index.html [accessed 7 January 2016].

Table 1. Main characteristics and serum levels of total effective xenoestrogen burden (TEXB) and specific organohalogenated compounds in breast cancer cases and controls ($n = 382$).

Characteristic	Controls	Breast cancer cases	p-Value[a]
No. of women	196	186	
Province			
Madrid	84 (42.9)	71 (38.2)	
Barcelona	34 (17.3)	33 (17.7)	
Navarra	26 (13.3)	27 (14.5)	
Cantabria	52 (26.5)	55 (29.6)	
Age (years)	59.8 ± 10.7	59.7 ± 11.1	
Body mass index[b] (kg/m^2)	26.2 ± 4.5	26.4 ± 4.5	
Education level			0.40
Primary or less	94 (48.0)	102 (54.8)	
High school	72 (36.7)	59 (31.7)	
College	30 (15.3)	25 (13.5)	
Smoking status[b]			0.28
Never	124 (63.3)	103 (55.6)	
Former	33 (16.8)	41 (22.2)	
Current	39 (19.9)	41 (22.2)	
No. of births			0.38
Nulliparous	33 (16.9)	39 (21.1)	
1–2	105 (53.9)	102 (55.1)	
≥ 3	57 (29.2)	44 (23.8)	
Age at first birth[c] (years)	26.5 ± 4.4	26.7 ± 5.4	0.81
Menopausal status			0.43
Premenopausal	28 (14.3)	32 (17.2)	
Postmenopausal	168 (85.7)	154 (82.8)	
Use of hormone replacement therapy			0.46
Never	177 (94.7)	167 (92.8)	
Ever	10 (5.3)	13 (7.2)	
Previous breast biopsy			0.02
No	189 (96.4)	168 (90.8)	
Yes	7 (3.6)	17 (9.2)	
Family history of breast cancer			0.27
No	166 (84.6)	147 (79.0)	
Second-degree relative	15 (7.7)	16 (8.6)	
First-degree relative	15 (7.7)	23 (12.4)	
Serum total lipids (mg/mL)	7.67 ± 1.87	7.42 ± 1.60	0.17
Serum TEXB-α[d] (Eeq pM/mL)	2.99 (7.86)	8.32 (5.72)	< 0.001
Undetermined estrogenicity	90 (45.9)	79 (42.5)	0.50
Serum TEXB-β[d] (Eeq pM/mL)	5.96 (5.65)	9.94 (4.57)	0.01
Undetermined estrogenicity	70 (35.7)	64 (34.4)	0.79

Serum PCB-138[e] (ng/mL)	0.89 (3.25)	1.04 (3.13)	0.21
Serum PCB-153[e] (ng/mL)	1.37 (3.28)	1.62 (2.92)	0.15
Serum PCB-180[e] (ng/mL)	0.72 (3.07)	0.71 (2.71)	0.97
Serum HCB[e] (ng/mL)	0.68 (3.40)	0.53 (4.11)	0.06
Serum *p,p*'-DDE[e] (ng/mL)	2.69 (5.04)	2.45 (4.42)	0.56

Values are means ± SDs or numbers (percentages).
[a]*p*-Value for homogeneity of means or proportions between breast cancer cases and controls. [b]Body mass index and smoking status one year before baseline interview. [c]Age at first birth among parous women. [d]Geometric mean (geometric SD) serum levels of the total effective xenoestrogen burden of alpha (TEXB-α) and beta (TEXB-β) fractions, together with numbers (percentages) of samples with undetermined estrogenicity in the bioassay. [e]Geometric mean (geometric SD) serum concentrations of polychlorinated biphenyl congeners 138 (PCB-138), 153 (PCB-153), 180 (PCB-180), hexachlorobenzene (HCB), and *p,p*'-dichlorodiphenyldichloroethylene (*p,p*'-DDE).

Table 2. Main characteristics and serum concentrations of specific organohalogenated compounds by tertile of total effective xenoestrogen burden of alpha (TEXB-α) and beta (TEXB-β) fractions among controls (*n* = 196).

Characteristic	Serum TEXB-α[a] (Eeq pM/mL)						Serum TEXB-β[a] (Eeq pM/mL)					
	Tertile 1 (≤ 2.62)	Tertile 2 (2.63–8.75)	Tertile 3 (≥ 8.76)	*p* for trend[b]	Undetermined estrogenicity	*p*-Value[c]	Tertile 1 (≤ 4.56)	Tertile 2 (4.57–11.27)	Tertile 3 (≥ 11.28)	*p* for trend[b]	Undetermined estrogenicity	*p*-Value[c]
No. of control women	35	36	35		90		42	42	42		70	
Median serum level (Eeq pM/mL)	0.07	4.78	15.18				2.29	7.41	19.89			
Province				0.002		< 0.001				0.08		< 0.001
Madrid	31.4	61.1	48.6		37.8		47.6	50.0	61.9		24.3	
Barcelona	17.2	2.8	14.3		24.4		11.9	14.3	2.4		31.4	
Navarra	11.4	27.8	25.7		3.3		19.1	11.9	28.6		1.4	
Cantabria	40.0	8.3	11.4		34.5		21.4	23.8	7.1		42.9	
Age (years)	57.0	61.8	58.9	0.73	60.5	0.40	57.7	60.6	59.9	0.46	60.7	0.40
Body mass index (kg/m^2)	27.5	25.3	26.5	0.65	25.9	0.39	26.0	25.5	26.5	0.50	26.4	0.54
High school education or more	62.9	44.4	54.3	0.68	50.0	0.60	57.1	52.4	59.5	0.72	44.3	0.11
Ever smoker	45.7	36.1	37.1	0.55	33.3	0.36	35.7	40.5	38.1	0.90	34.3	0.60
Nulliparous	22.9	11.1	14.3	0.45	18.0	0.72	14.3	19.0	7.1	0.21	23.2	0.09
Age at first birth (years)	26.1	26.9	26.8	0.70	26.4	0.74	26.8	27.0	27.0	0.88	25.7	0.08
Postmenopausal	71.4	94.4	88.6	0.11	86.7	0.73	81.0	85.7	92.9	0.10	84.3	0.67
Ever use of hormone therapy	3.1	3.0	2.9	0.95	8.0	0.12	7.1	2.4	7.1	0.80	4.8	0.83
Previous breast biopsy	2.9	5.6	0.0	0.30	4.4	0.54	7.1	4.8	0.0	0.05	2.9	0.68
Family history of breast cancer	25.7	16.7	17.1	0.45	10.0	0.05	16.7	19.0	21.4	0.59	8.6	0.04
Serum total lipids (mg/mL)	7.73	7.36	7.93	0.51	7.66	0.97	7.35	8.04	7.82	0.47	7.54	0.50
Serum PCB-138[d] (ng/mL)	0.98	0.77	0.58	0.11	1.09	0.03	0.73	0.95	0.66	0.55	1.17	0.02
Serum PCB-153[d] (ng/mL)	1.16	1.51	1.27	0.91	1.45	0.56	1.18	1.88	1.11	0.53	1.40	0.83
Serum PCB-180[d] (ng/mL)	0.61	0.42	0.60	0.82	1.01	< 0.001	0.56	0.69	0.53	0.67	1.03	0.001
Serum HCB[d] (ng/mL)	0.62	0.56	0.45	0.30	0.90	0.003	0.53	0.83	0.42	0.19	0.93	0.007
Serum *p,p*'-DDE[d] (ng/mL)	2.69	1.37	1.54	0.26	4.37	< 0.001	1.45	3.08	1.89	0.82	4.42	0.001

Values are means or percentages.
[a]Tertiles of the total effective xenoestrogen burden of alpha (TEXB-α) and beta (TEXB-β) fractions, together with serum samples with undetermined estrogenicity in the bioassay. [b]*p*-Value for linear trend in means or proportions across tertiles of the total effective xenoestrogen burden of alpha and beta fractions; except for province, which corresponds to the *p*-value for homogeneity of province distributions among tertiles. [c]*p*-Value for homogeneity of means or

25

proportions comparing serum samples with determined estrogenicity in the bioassay (all tertiles combined) with those undetermined. [d]Geometric mean serum concentrations of polychlorinated biphenyl congeners 138 (PCB-138), 153 (PCB-153), 180 (PCB-180), hexachlorobenzene (HCB), and *p,p*'-dichlorodiphenyldichloroethylene (*p,p*'-DDE).

Table 3. Odds ratios for breast cancer (95% confidence intervals) by tertile of total effective xenoestrogen burden of alpha (TEXB-α) and beta (TEXB-β) fractions (*n* = 382).

TEXB	Tertile 1	Tertile 2	Tertile 3	*p* for trend[a]	Undetermined estrogenicity[b]
Serum TEXB-α[c] (Eeq pM/mL)	≤ 2.62	2.63–8.75	≥ 8.76		
No. of controls/breast cancer cases	35/18	36/32	35/57		90/79
Model 1[d]	1.00 (reference)	1.64 (0.74, 3.62)	3.04 (1.38, 6.70)	0.005	0.83 (0.52, 1.32)
Model 2[e]	1.00 (reference)	1.77 (0.76, 4.10)	3.45 (1.50, 7.97)	0.003	0.73 (0.45, 1.20)
Model 3[f]	1.00 (reference)	1.50 (0.55, 4.08)	1.80 (0.63, 5.09)	0.32	
Serum TEXB-β[c] (Eeq pM/mL)	≤ 4.56	4.57–11.27	≥ 11.28		
No. of controls/breast cancer cases	42/21	42/43	42/58		70/64
Model 1[d]	1.00 (reference)	2.14 (1.06, 4.35)	3.27 (1.62, 6.61)	0.002	0.86 (0.52, 1.41)
Model 2[e]	1.00 (reference)	2.35 (1.10, 5.03)	4.01 (1.88, 8.56)	0.001	0.97 (0.58, 1.65)
Model 3[f]	1.00 (reference)	1.75 (0.65, 4.71)	3.53 (1.24, 10.0)	0.02	

[a]*p*-Value for linear risk trend across tertiles based on an ordinal variable with the median level of each tertile. [b]Odds ratio for breast cancer comparing women with undetermined estrogenicity in the bioassay with all other women with determined estrogenicity. [c]Serum levels of the total effective xenoestrogen burden of alpha (TEXB-α) and beta (TEXB-β) fractions. [d]Adjusted for province (Madrid, Barcelona, Navarra, or Cantabria), age (continuous), body mass index (continuous), education level (primary or less, high school, or college), and serum total lipid levels (continuous). [e]Further adjusted for smoking status (never, former, or current), number of births (nulliparous, 1–2, or ≥ 3), age at first birth (continuous), menopausal status (premenopausal or postmenopausal), use of hormone replacement therapy (never or ever), previous breast biopsy (no or yes), and family history of breast cancer (no, second-degree relative, or first-degree relative). [f]Further adjusted for the other fraction of total effective xenoestrogen burden (tertiles).

Table 4. Odds ratios for breast cancer (95% confidence intervals) by tertile of specific organohalogenated compounds ($n = 382$).

Organohalogenated compound	Tertile 1	Tertile 2	Tertile 3	p for trend[a]
Serum PCB-138[b] (ng/mL)	≤ 0.80	0.81–1.59	≥ 1.60	
No. of controls/breast cancer cases	65/52	65/60	66/74	
Model 1[c]	1.00 (reference)	1.27 (0.75, 2.14)	1.63 (0.93, 2.85)	0.09
Model 2[d]	1.00 (reference)	1.30 (0.74, 2.27)	1.73 (0.96, 3.14)	0.07
Model 3[e]	1.00 (reference)	1.34 (0.64, 2.81)	1.64 (0.78, 3.46)	0.20
Serum PCB-153[b] (ng/mL)	≤ 0.90	0.91–2.07	≥ 2.08	
No. of controls/breast cancer cases	63/50	68/78	65/58	
Model 1[c]	1.00 (reference)	1.54 (0.92, 2.58)	1.21 (0.69, 2.12)	0.85
Model 2[d]	1.00 (reference)	1.42 (0.83, 2.42)	1.36 (0.75, 2.45)	0.46
Model 3[e]	1.00 (reference)	0.90 (0.45, 1.82)	1.33 (0.64, 2.75)	0.36
Serum PCB-180[b] (ng/mL)	≤ 0.52	0.53–1.17	≥ 1.18	
No. of controls/breast cancer cases	65/63	66/56	65/67	
Model 1[c]	1.00 (reference)	0.82 (0.48, 1.41)	1.04 (0.59, 1.85)	0.73
Model 2[d]	1.00 (reference)	0.82 (0.46, 1.43)	1.01 (0.55, 1.87)	0.81
Model 3[e]	1.00 (reference)	0.96 (0.47, 1.98)	1.09 (0.49, 2.43)	0.81
Serum HCB[b] (ng/mL)	≤ 0.43	0.44–1.25	≥ 1.26	
No. of controls/breast cancer cases	65/75	66/58	65/53	
Model 1[c]	1.00 (reference)	0.69 (0.41, 1.15)	0.56 (0.30, 1.02)	0.09
Model 2[d]	1.00 (reference)	0.69 (0.41, 1.18)	0.60 (0.32, 1.15)	0.18
Model 3[e]	1.00 (reference)	0.63 (0.32, 1.24)	0.64 (0.27, 1.50)	0.38
Serum *p,p*'-DDE[b] (ng/mL)	≤ 1.37	1.38–6.76	≥ 6.77	
No. of controls/breast cancer cases	65/56	66/86	65/44	
Model 1[c]	1.00 (reference)	1.50 (0.90, 2.49)	0.72 (0.40, 1.31)	0.06
Model 2[d]	1.00 (reference)	1.59 (0.94, 2.70)	0.84 (0.45, 1.58)	0.20

Model 3[e]	1.00 (reference)	1.61 (0.81, 3.21)	0.63 (0.27, 1.46)	0.10

[a]p-Value for linear risk trend across tertiles based on an ordinal variable with the median level of each tertile. [b]Serum concentrations of polychlorinated biphenyl congeners 138 (PCB-138), 153 (PCB-153), 180 (PCB-180), hexachlorobenzene (HCB), and p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE). [c]Adjusted for province (Madrid, Barcelona, Navarra, or Cantabria), age (continuous), body mass index (continuous), education level (primary or less, high school, or college), and serum total lipid levels (continuous). [d]Further adjusted for smoking status (never, former, or current), number of births (nulliparous, 1–2, or ≥ 3), age at first birth (continuous), menopausal status (premenopausal or postmenopausal), use of hormone replacement therapy (never or ever), previous breast biopsy (no or yes), and family history of breast cancer (no, second-degree relative, or first-degree relative). [e]Further adjusted for the total effective xenoestrogen burden of beta fraction (tertiles).

Figure legends

Figure 1. Odds ratios for breast cancer by serum levels of total effective xenoestrogen burden of alpha (*A*) and beta fractions (*B*).

Curves represent adjusted odds ratios based on restricted quadratic splines for log-transformed levels of total effective xenoestrogen burden of alpha and beta fractions with knots at the 10th, 50th, and 95th percentiles. The reference value (odds ratio = 1) was set at the 20th percentile of each fraction distribution among controls (0.54 and 2.97 Eeq pM/mL for alpha and beta fractions, respectively). Odd ratios were adjusted for province, age, body mass index, education level, serum total lipid levels, smoking status, number of births, age at first birth, menopausal status, use of hormone replacement therapy, previous breast biopsy, and family history of breast cancer (bold curves), and further adjusted for the other fraction of total effective xenoestrogen burden (thin curves). Histograms represent each fraction distribution among controls (shaded bars) and breast cancer cases (white bars).

Figure 2. Odds ratios for breast cancer comparing the third with the first tertile of total effective xenoestrogen burden of alpha (TEXB-α) and beta (TEXB-β) fractions by subgroup.

Subgroup-specific odds ratios (squares with area inversely proportional to the variance) and their 95% confidence intervals (horizontal lines) were obtained from logistic regression models with interaction terms of serum TEXB-α and TEXB-β tertiles with the corresponding subgroup indicators and adjusted for province, age, body mass index, education level, serum total lipid levels, smoking status, number of births, age at first birth, menopausal status, use of hormone replacement therapy, previous breast biopsy, and family history of breast cancer.

Figure 3. Diagram with causal relations among total effective xenoestrogen burden of alpha (TEXB-α) and beta (TEXB-β) fractions, breast cancer, and unmeasured exposures to xenoestrogens and endogenous hormones.

The causal path from TEXB-α to breast cancer $X{\to}Y$ is confounded by the undirected path $X{\leftarrow}U_1{\to}Z{\to}Y$, which can be blocked by adjusting for TEXB-β. However, conditioning on TEXB-β unblocks the other undirected path $X{\leftarrow}U_1{\to}Z{\leftarrow}U_2{\to}Y$, thus resulting in selection bias.

Figure 1.

Figure 2.

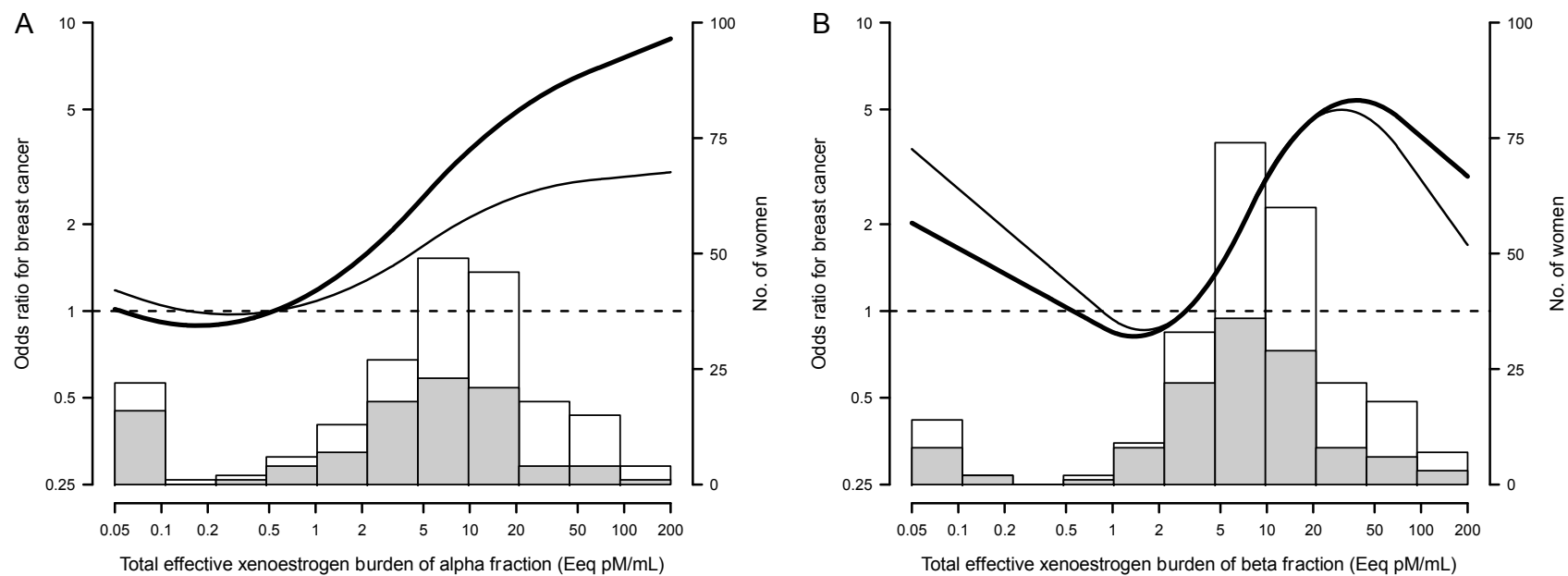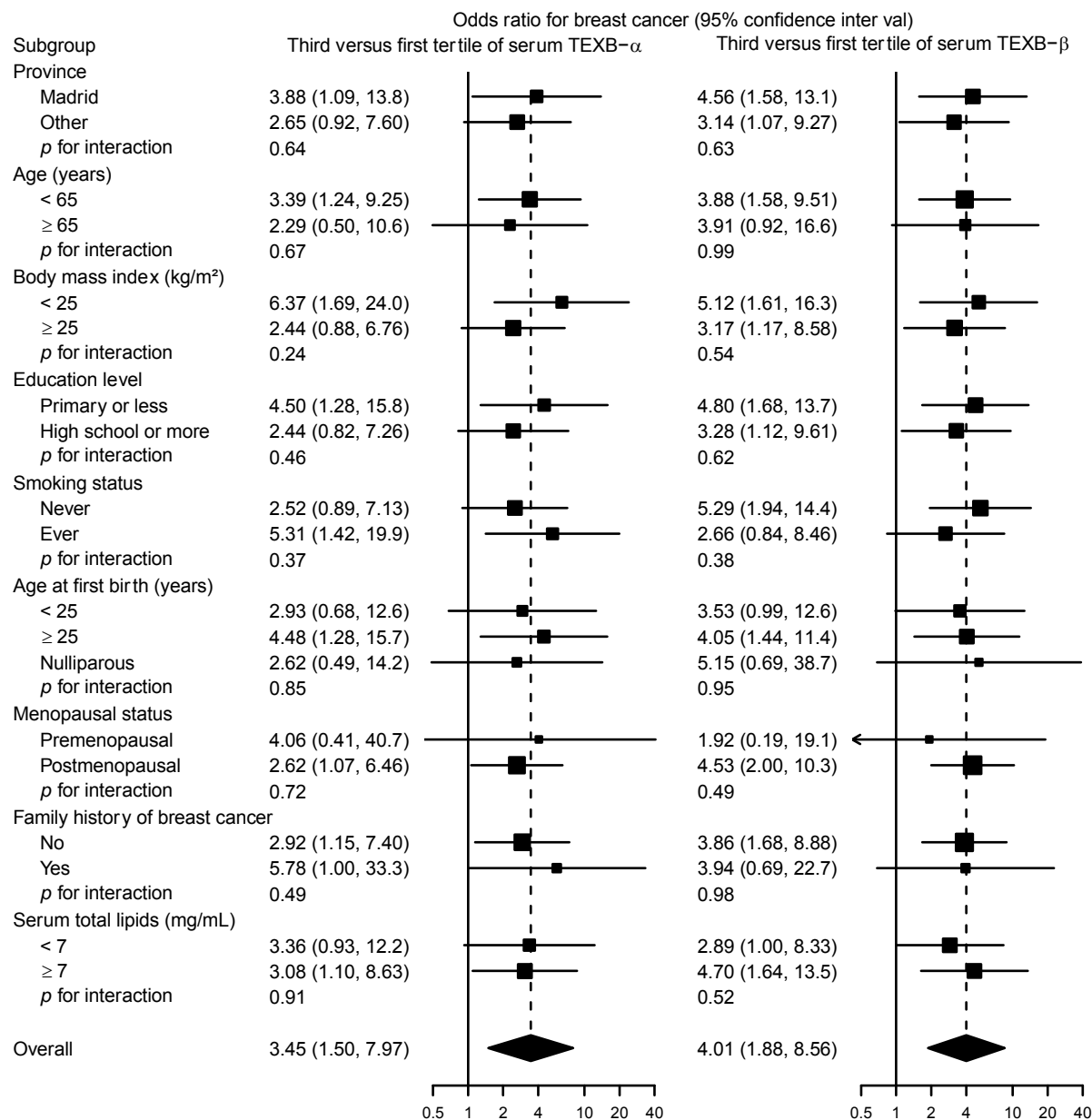Odds ratio for breast cancer (95% confidence interval)

Subgroup	Third versus first tertile of serum TEXB-α		Third versus first tertile of serum TEXB-β	
Province				
Madrid	3.88 (1.09, 13.8)		4.56 (1.58, 13.1)	
Other	2.65 (0.92, 7.60)		3.14 (1.07, 9.27)	
p for interaction	0.64		0.63	
Age (years)				
< 65	3.39 (1.24, 9.25)		3.88 (1.58, 9.51)	
≥ 65	2.29 (0.50, 10.6)		3.91 (0.92, 16.6)	
p for interaction	0.67		0.99	
Body mass index (kg/m²)				
< 25	6.37 (1.69, 24.0)		5.12 (1.61, 16.3)	
≥ 25	2.44 (0.88, 6.76)		3.17 (1.17, 8.58)	
p for interaction	0.24		0.54	
Education level				
Primary or less	4.50 (1.28, 15.8)		4.80 (1.68, 13.7)	
High school or more	2.44 (0.82, 7.26)		3.28 (1.12, 9.61)	
p for interaction	0.46		0.62	
Smoking status				
Never	2.52 (0.89, 7.13)		5.29 (1.94, 14.4)	
Ever	5.31 (1.42, 19.9)		2.66 (0.84, 8.46)	
p for interaction	0.37		0.38	
Age at first birth (years)				
< 25	2.93 (0.68, 12.6)		3.53 (0.99, 12.6)	
≥ 25	4.48 (1.28, 15.7)		4.05 (1.44, 11.4)	
Nulliparous	2.62 (0.49, 14.2)		5.15 (0.69, 38.7)	
p for interaction	0.85		0.95	
Menopausal status				
Premenopausal	4.06 (0.41, 40.7)		1.92 (0.19, 19.1)	
Postmenopausal	2.62 (1.07, 6.46)		4.53 (2.00, 10.3)	
p for interaction	0.72		0.49	
Family history of breast cancer				
No	2.92 (1.15, 7.40)		3.86 (1.68, 8.88)	
Yes	5.78 (1.00, 33.3)		3.94 (0.69, 22.7)	
p for interaction	0.49		0.98	
Serum total lipids (mg/mL)				
< 7	3.36 (0.93, 12.2)		2.89 (1.00, 8.33)	
≥ 7	3.08 (1.10, 8.63)		4.70 (1.64, 13.5)	
p for interaction	0.91		0.52	
Overall	3.45 (1.50, 7.97)		4.01 (1.88, 8.56)	

0.5 1 2 4 10 20 40   0.5 1 2 4 10 20 40

Figure 3.

U_1: Xenoestrogen exposure

U_2: Endogenous hormones

Z: TEXB-β

X: TEXB-α

Y: Breast cancer